



**PLURIPOTENT EMBRYONIC-LIKE STEM CELLS, COMPOSITIONS,
METHODS AND USES THEREOF**

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FIELD OF THE INVENTION

This invention relates generally to pluripotent stem cells, particularly to embryonic-
10 like pluripotent stem cells. The invention also relates to uses of the stem cells for
tissue engineering in cell or tissue transplantation, in gene therapy, and in identifying,
assaying or screening with respect to cell-cell interactions, lineage commitment,
development genes and growth or differentiation factors.

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BACKGROUND OF THE INVENTION

The formation of tissues and organs occurs naturally during prenatal development.
The development of multicellular organisms follows pre-determined molecular and
cellular pathways culminating in the formation of entities composed of billions of
20 cells with defined functions. Cellular development is accomplished through cellular
proliferation, lineage-commitment, and lineage-progression, resulting in the formation
of differentiated cell types. This process begins with the totipotent zygote and
continues throughout the life of the individual. As development proceeds from the
totipotent zygote, cells proliferate and segregate by lineage-commitment into the
25 pluripotent primary germ layers, ectoderm, mesoderm, and endoderm. Further
segregation of these germ layers through progressive lineage-commitment into
progenitor (multipotent, tripotent, bipotent and eventually unipotent) lineages further
defines the differentiation pathways of the cells and their ultimate function.

30 Development proceeds from the fertilized egg, to formation of a blastula and then a
gastrula. Gastrulation is the process by which the bilaminar embryonic disc is
converted into a trilaminar embryonic disc. Gastrulation is the beginning of

morphogenesis or development of the body form. gastrulation begins with the formation of the primitive streak on the surface of the epiblast of the embryonic disk. Formation of the primitive streak, germ layers, and notochord are the important processes occurring during gastrulation. Each of the three germ layers - ectoderm, 5 endoderm, and mesoderm- gives rise to specific tissues and organs.

The organization of the embryo into three layers roughly corresponds to the organization of the adult, with gut on the inside, epidermis on the outside, and connective tissue in between. The endoderm is the source of the epithelial linings of 10 the respiratory passages and gastrointestinal tract and gives rise to the pharynx, esophagus, stomach, intestine and to many associated glands, including salivary glands, liver, pancreas and lungs. The mesoderm gives rise to smooth muscular coats, connective tissues, and vessels associated with the tissues and organs; mesoderm also forms most of the cardiovascular system and is the source of blood cells and bone 15 marrow, the skeleton, striated muscles, and the reproductive and excretory organs. Ectoderm will form the epidermis (epidermal layer of the skin), the sense organs, and the entire nervous system, including brain, spinal cord, and all the outlying components of the nervous system.

20 While a majority of the cells progress through the sequence of development and differentiation, a few cells leave this pathway to become reserve stem cells that provide for the continual maintenance and repair of the organism. Reserve stem cells include progenitor stem cells and pluripotent stem cells. Progenitor cells (e.g., precursor stem cells, immediate stem cells, and forming or -blast cells, e.g., 25 myoblasts, adipoblasts, chondroblasts, etc.) are lineage-committed. Unipotent stem cells will form tissues restricted to a single lineage (such as the myogenic, fibrogenic, adipogenic, chondrogenic, osteogenic lineages, etc.). Bipotent stem cells will form tissues belonging to two lineages (such as the chondro-osteogenic, adipo-fibroblastic lineages, etc.). Tripotent stem cells will form tissues belonging to three lineages (such 30 as chondro-osteo-adipogenic lineage, etc.). Multipotent stem cells will form multiple cell types within a lineage (such as the hematopoietic lineage). Progenitor stem cells

will form tissues limited to their lineage, regardless of the inductive agent that may be added to the medium. They can remain quiescent. Lineage-committed progenitor cells are capable of self-replication but have a limited life-span (approximately 50-70 cell doublings) before programmed cell senescence occurs. They can also be 5 stimulated by various growth factors to proliferate. If activated to differentiate, these cells require progression factors (i.e., insulin, insulin-like growth factor-I, and insulin-like growth factor-II) to stimulate phenotypic expression.

In contrast, pluripotent cells are lineage-uncommitted, i.e., they are not committed to 10 any particular tissue lineage. They can remain quiescent. They can also be stimulated by growth factors to proliferate. If activated to proliferate, pluripotent cells are capable of extended self-renewal as long as they remain lineage-uncommitted. Pluripotent cells have the ability to generate various lineage-committed progenitor cells from a single clone at any time during their life span. For example, a prenatal pluripotent 15 mouse clone after more than 690 doublings (Young et al 1998a) and a postnatal pluripotent rat clone after more than 300 doublings (Young et al 1999) were both induced to form lineage-committed progenitor cells that after long term dexamethasone exposure, went on to differentiate into skeletal muscle, fat, cartilage, that exhibited characteristic morphological and phenotypic expression markers. This 20 lineage-commitment process necessitates the use of either general (e.g., dexamethasone) or lineage-specific (e.g., bone morphogenetic protein-2, muscle morphogenetic protein, etc.) commitment induction agents. Once pluripotent cells are induced to commit to a particular tissue lineage, they assume the characteristics of lineage-specific progenitor cells. They can remain quiescent or they can proliferate. 25 under the influence of specific inductive agents. Their ability to replicate is limited to approximately 50-70 cell doublings before programmed cell senescence occurs and they require the assistance of progression factors to stimulate phenotypic expression.

Embryonic stem cells are uncommitted, totipotent cells isolated from embryonic 30 tissue. When injected into embryos, they can give rise to all somatic lineages as well as functional gametes. In the undifferentiated state these cells are alkaline

phosphatase-positive, express immunological markers for embryonic stem and embryonic germ cells, are telomerase positive, and show capabilities for extended self-renewal. Upon differentiation these cells express a wide variety of cell types, derived from ectodermal, mesoderm, and endodermal embryonic germ layers.

5 Embryonic stem (ES) cells have been isolated from the blastocyst, inner cell mass or gonadal ridges of mouse, rabbit, rat, pig, sheep, primate and human embryos (Evans and Kauffman, 1981; Iannaccone et al., 1994; Graves and Moreadith, 1993; Martin, 1981; Notarianni et al., 1991; Thomson, et al., 1995; Thomson, et al., 1998; Shambrook, et al., 1998).

10 ES cells are used for both *in vitro* and *in vivo* studies. ES cells retain their capacity for multilineage differentiation during genetic manipulation and clonal expansion. The uncommitted cells provide a model system from which to study cellular differentiation and development and provide a powerful tool for genome manipulation, e.g. when used as vectors to carry specific mutations into the genome (particularly the mouse genome) by homologous recombination (Brown et al., 1992). While ES cells are a potential source of cells for transplantation studies, these prospects have been frustrated by the disorganized and heterogeneous nature of development in culture, stimulating the necessary development of strategies for

15 manipulation, e.g. when used as vectors to carry specific mutations into the genome (particularly the mouse genome) by homologous recombination (Brown et al., 1992). While ES cells are a potential source of cells for transplantation studies, these prospects have been frustrated by the disorganized and heterogeneous nature of development in culture, stimulating the necessary development of strategies for

20 selection of lineage-restricted precursors from differentiating populations (Li et al., 1998). ES cells implanted into animals or presented subcutaneously form teratomas-tumors containing various types of tissues containing derivatives of all three germ layers (Thomson et al., 1988).

25 Examples of progenitor and pluripotent stem cells from the mesodermal germ layer include the unipotent myosatellite myoblasts of muscle (Mauro, 1961; Campion, 1984; Grounds et al., 1992); the unipotent adipoblast cells of adipose tissue (Ailhaud et al., 1992); the unipotent chondrogenic cells and osteogenic cells of the perichondrium and periosteum, respectively (Cruess, 1982; Young et al., 1995); the

30 bipotent adipofibroblasts of adipose tissue (Vierck et al., 1996); the bipotent chondrogenic/osteogenic stem cells of marrow (Owen, 1988; Beresford, 1989;

Rickard et al., 1994; Caplan et al., 1997; Prockop, 1997); the tripotent chondrogenic/osteogenic/adipogenic stem cells of marrow (Pittenger et al., 1999); the multipotent hematopoietic stem cells of marrow (Palis and Segel, 1998; McGuire, 1998; Ratajczak et al., 1998); the multipotent cardiogenic hematopoietic/endotheliogenic cells of marrow (Eisenberg and Markwald, 1997); and the pluripotent mesenchymal stem cells of the connective tissues (Young et al., 1993, 1998a; Rogers et al., 1995).

Pluripotent mesenchymal stem cells and methods of isolation and use thereof are described in United States Patent No. 5,827,735, issued October 27, 1998, which is hereby incorporated by reference in its entirety. Such pluripotent mesenchymal stem cells are substantially free of lineage-committed cells and are capable of differentiating into multiple tissues of mesodermal origin, including but not limited to bone, cartilage, muscle, adipose tissue, vasculature, tendons, ligaments and hematopoietic. Further compositions of such pluripotent mesenchymal stem cells and the particular use of pluripotent mesenchymal stem cells in cartilage repair are described in United States Patent No. 5,906,934, issued May 25, 1999, which is hereby incorporated by reference in its entirety.

Progenitor or pluripotent stem cell populations having mesodermal lineage capability have been isolated from multiple animal species, e.g., avians (Young et al., 1992a, 1993, 1995), mice (Rogers et al., 1995; Saito et al., 1995; Young et al., 1998a), rats (Grigoriadis et al., 1988; Lucas et al., 1995, 1996; Dixon et al., 1996; Warejcka et al., 1996), rabbits (Pate et al., 1993; Wakitani et al., 1994; Grande et al., 1995; Young, R.G. et al., 1998), and humans (Caplan et al., 1993; Young, 1999a-c). Clonogenic analysis (isolation of individual clones by repeated limiting serial dilution) from populations of mesodermal stem cells isolated from prenatal chicks (Young et al., 1993) and prenatal mice (Rogers et al., 1995; Young et al., 1998a) revealed two categories of cells: lineage-committed progenitor cells and lineage-uncommitted pluripotent cells. Non-immortalized progenitor cells are capable of self-replication but have a finite life-span limited to approximately 50-70 cell doublings before programmed cell senescence occurs. They can remain quiescent or be induced to

proliferate, progress down their lineage pathway, and/or differentiate. One unique characteristic of progenitor cells is that their phenotypic expression can be accelerated by treatment with progression factors such as insulin, insulin-like growth factor-I (IGF-I), or insulin-like growth factor-II (IGF-II) (Young et al., 1993, 1998a,b; Young, 5 1999a; Rogers et al., 1995).

Progenitor cells are lineage-committed and lineage-restricted. They can remain quiescent or be induced to proliferate, progress down their lineage pathway, and/or differentiate by treatment with appropriate bioactive factors (Young et al., 1998b). By 10 contrast, pluripotent mesenchymal stem cells PPMSCs were found to be lineage-uncommitted and lineage-unrestricted, with respect to the mesodermal germ layer. PPMSCs from prenatal animals were capable of extended self-renewal as long as they remain uncommitted to a particular lineage. Once PPMSCs commit to a particular tissue lineage they assume the characteristics of progenitor cells for that lineage and 15 their ability to replicate is limited to approximately 50-70 cell doublings before programmed cell senescence occurred. PPMSCs could remain quiescent, and if not, appropriate bioactive factors were necessary to induce proliferation, lineage-commitment, lineage-progression, and/or differentiation of stem cells (Young et al., 1998b).

20 The formation of tissues and organs occurs naturally in early normal human development, however, the ability to regenerate most human tissues damaged or lost due to trauma or disease is substantially diminished in adults. Every year millions of Americans suffer tissue loss or end-stage organ failure. The total national health care 25 costs for these patients exceeds 400 billion dollars per year. Currently over 8 million surgical procedures are performed annually in the United States to treat these disorders and 40 to 90 million hospital days are required. Although these therapies have saved and improved countless lives, they remain imperfect solutions. Options such as tissue transplantation and surgical intervention are severely limited by a 30 critical donor shortage and possible long term morbidity. Indeed, donor shortages

worsen every year and increasing numbers of patients die while on waiting lists for needed organs (Langer and Vicanti, 1993).

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function (Langer and Vicanti, 1993). Three general strategies have been adopted for the creation of new tissue: (1). Isolated cells or cell substitutes applied to the area of tissue deficiency or compromise. (2). Cells placed on or within matrices. In closed systems, cells are isolated from the body by a membrane allowing permeation of nutrients and wastes while excluding large entities such as antibodies or immune cells from destroying the implant. In open systems, cells attached to matrices are implanted and become incorporated into the body. (3). Tissue-inducing substances, that rely on growth factors to regulate specific cells to a committed pattern of growth resulting in tissue regeneration, and methods to deliver these substances to their targets.

Based on available evidence, a wide variety of transplants, congenital malformations, elective surgeries, diseases, and genetic disorders have the potential for treatment with pluripotent stem cells, alone or in combination with morphogenetic proteins, growth factors, genes, and/or controlled-release delivery systems. A preferred treatment is the treatment of tissue loss where the object is to increase the number of cells available for transplantation, thereby recreating the missing tissue (i.e., tissue loss, congenital malformations, breast reconstruction, blood transfusions, or muscular dystrophy) or providing sufficient numbers of cells for ex vivo gene therapy (muscular dystrophy).
The expected benefit using pluripotent stem cells, is its potential for unlimited proliferation prior to (morphogenetic protein-induced) commitment to a particular tissue lineage and then once committed as a progenitor stem cell, an additional fifty to seventy doublings before programmed cell senescence. These proliferative attributes are very important when limited amounts of tissue are available for transplantation.
Tissue loss may result from acute injuries as well as surgical interventions, i.e., amputation, tissue debridement, and surgical extirpations with respect to cancer.

traumatic tissue injury, congenital malformations, vascular compromise, elective surgeries, etc. and account for approximately 3.5 million operations per year in the United States.

5 The expected benefits from the use of various pluripotent stem cells can be illustrated in considering, for example, applications of pluripotent mesenchymal stem cells. Pluripotent mesenchymal stem cells can be utilized for the replacement of potentially multiple tissues of mesodermal origin (i.e., bone, cartilage, muscle, adipose tissue, vasculature, tendons, ligaments and hematopoietic). Such tissues generated, for
10 instance, ex vivo with specific morphogenetic proteins and growth factors to recreate the lost tissues. The recreated tissues would then be transplanted to repair the site of tissue loss. An alternative strategy could be to provide pluripotent stem cells, as cellular compositions or incorporated, for instance, into matrices, transplant into the area of need, and allow endogenous morphogenetic proteins and growth factors to
15 induce the pluripotent stem cells to recreate the missing histoarchitecture of the tissue. This approach is exemplified in U.S. Patent No. 5,903,934 which is incorporated herein in its entirety, which describes the implanting of pluripotent mesenchymal stem cells into a polymeric carrier, to provide differentiation into cartilage and/or bone at a site for cartilage repair.

20 The identification of an additional tissue source for transplantation therapies, that (a) can be isolated and sorted; (b) has unlimited proliferation capabilities while retaining pluripotency; (c) can be manipulated to commit to multiple separate tissue lineages; (d) is capable of incorporating into the existing tissue; and (d) can subsequently
25 express the respective differentiated tissue type, may prove beneficial to therapies that maintain or increase the functional capacity and/or longevity of lost, damaged, or diseased tissues.

30 The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

In its broadest aspect, the present invention extends to an stem cell, derived from non-embryonic animal cells or tissue, capable of self regeneration and capable of 5 differentiation to cells of endodermal, ectodermal and mesodermal lineages.

In a particular aspect, the present invention extends to an pluripotent embryonic-like stem cell, derived from postnatal animal cells or tissue, capable of self regeneration and capable of differentiation to cells of endodermal, ectodermal and mesodermal 10 lineages.

In a further aspect, the present invention extends to an pluripotent embryonic-like stem cell, derived from adult animal cells or tissue, capable of self regeneration and capable of differentiation to cells of endodermal, ectodermal and mesodermal 15 lineages.

The pluripotent embryonic-like stem cell of the present invention may be isolated from non-human cells or human cells.

20 The pluripotent embryonic-like stem cell of the present invention may be isolated from the non-embryonic tissue selected from the group of muscle, dermis, fat, tendon, ligament, perichondrium, periosteum, heart, aorta, endocardium, myocardium, epicardium, large arteries and veins, granulation tissue, peripheral nerves, peripheral ganglia, spinal cord, dura, leptomeninges, trachea, esophagus, stomach, small 25 intestine, large intestine, liver, spleen, pancreas, parietal peritoneum, visceral peritoneum, parietal pleura, visceral pleura, urinary bladder, gall bladder, kidney, associated connective tissues or bone marrow.

This invention further relates to cells, particularly pluripotent or progenitor cells, 30 which are derived from the pluripotent embryonic-like stem cell. The cells may be lineage-committed cells, which cells may be committed to the endodermal,

ectodermal or mesodermal lineage. For instance, a lineage-committed cell of the mesodermal lineage, for instance an adipogenic, myogenic or chondrogenic progenitor cell may be derived from the pluripotent embryonic-like stem cell.

5 The invention also relates to pluripotent cells derived from the pluripotent embryonic-like stem cells, including pluripotent mesenchymal stem cells, pluripotent endodermal stem cells and pluripotent ectodermal stem cells. Any such pluripotent cells are capable of self-renewal and differentiation.

10 In a further aspect, the present invention relates to a culture comprising:

- (a) Pluripotent embryonic-like stem cells, capable of self regeneration and capable of differentiation to cells of endodermal, ectodermal and mesodermal lineages; and
- (b) a medium capable of supporting the proliferation of said stem cells.

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Such stem cell containing cultures may further comprise a proliferation factor or lineage commitment factor. The stem cells of such cultures may be isolated from non-human cells or human cells.

20 The invention further relates to methods of isolating an pluripotent embryonic-like stem cell. In particular, a method of isolating an pluripotent embryonic-like stem cell of the present invention, comprises the steps of:

- (a) obtaining cells from a non-embryonic animal source;
- (b) slow freezing said cells in medium containing 7.5% (v/v) dimethyl sulfoxide until a final temperature of -80°C is reached; and
- (c) culturing the cells.

30 The invention further relates to methods of isolating an pluripotent embryonic-like stem cell. In particular, a method of isolating an pluripotent embryonic-like stem cell of the present invention, comprises the steps of:

- (a) obtaining cells from a postnatal animal source;

(b) slow freezing said cells in medium containing 7.5% (v/v) dimethyl sulfoxide until a final temperature of -80°C is reached; and

(c) culturing the cells.

5 The invention further relates to methods of isolating an pluripotent embryonic-like stem cell. In particular, a method of isolating an pluripotent embryonic-like stem cell of the present invention, comprises the steps of:

(a) obtaining cells from an adult animal source;

(b) slow freezing said cells in medium containing 7.5% (v/v) dimethyl sulfoxide until a final temperature of -80°C is reached; and

10 (c) culturing the cells.

The invention further relates to methods of isolating an pluripotent embryonic-like stem cell. In particular, a method of isolating an pluripotent embryonic-like stem cell of the present invention, comprises the steps of:

15 (a) obtaining cells from a non-embryonic animal source;

(b) filtering said cells through a 20 μ m filter;

(c) slow freezing said cells in medium containing 7.5% (v/v) dimethyl sulfoxide until a final temperature of -80°C is reached; and

20 (d) culturing the cells.

In a further aspect, the methods of isolating an pluripotent embryonic-like stem cell relate to methods whereby a clonal population of such stem cells is isolated, wherein a single pluripotent embryonic-like stem cell is first isolated and then further cultured and expanded to generate a clonal population. A single pluripotent embryonic-like stem cell may be isolated by means of limiting dilution or such other methods as are known to the skilled artisan.

25 Thus, the present invention also relates to a clonal pluripotent embryonic-like stem cell line developed by such method.

In a particular aspect, the present invention relates to pluripotent embryonic-like stem cells or populations of such cells which have been transformed or transfected and thereby contain and can express a gene or protein of interest. Thus, this invention includes pluripotent embryonic-like stem cells genetically engineered to express a 5 gene or protein of interest. In as much as such genetically engineered stem cells can then undergo lineage-committment, the present invention further encompasses lineage-committed cells, which are derived from a genetically engineered pluripotent embryonic-like stem cell, and which express a gene or protein of interest. The lineage-committed cells may be endodermal, ectodermal or mesodermal lineage-committed cells and may be pluripotent, such as a pluripotent mesenchymal stem cell, 10 or progenitor cells, such as an adipogenic or a myogenic cell.

The invention then relates to methods of producing a genetically engineered pluripotent embryonic-like stem cell comprising the steps of:

15 (a) transfected pluripotent embryonic-like stem cells with a DNA construct comprising at least one of a marker gene or a gene of interest;

(b) selecting for expression of the marker gene or gene of interest in the pluripotent embryonic-like stem cells;

(c) culturing the stem cells selected in (b).

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In a particular aspect, the present invention encompasses genetically engineered pluripotent embryonic-like stem cell(s), including human and non-human cells, produced by such method.

25 The present invention further relates to methods for detecting the presence or activity of an agent which is a lineage-commitment factor comprising the steps of:

A. contacting the pluripotent embryonic-like stem cells of the present invention with a sample suspected of containing an agent which is a lineage-commitment factor; and

30 B. determining the lineage of the so contacted cells by morphology, mRNA expression, antigen expression or other means;

wherein the lineage of the contacted cells indicates the presence or activity of a lineage-commitment factor in said sample.

The present invention also relates to methods of testing the ability of an agent, 5 compound or factor to modulate the lineage-commitment of a lineage uncommitted cell which comprises

- A. culturing the pluripotent embryonic- like stem cells of the present invention in a growth medium which maintains the stem cells as lineage uncommitted cells;
- 10 B. adding the agent, compound or factor under test; and
- C. determining the lineage of the so contacted cells by morphology, mRNA expression, antigen expression or other means.

The invention includes an assay system for screening of potential agents, compounds 15 or drugs effective to modulate the proliferation or lineage-commitment of the pluripotent embryonic-like stem cells of the present invention.

In a further such aspect, the present invention relates to an assay system for screening agents, compounds or factors for the ability to modulate the lineage-commitment of a 20 lineage uncommitted cell, comprising:

- A. culturing the pluripotent embryonic-like stem cells of the present invention in a growth medium which maintains the stem cells as lineage uncommitted cells;
- 25 B. adding the agent, compound or factor under test; and
- C. determining the lineage of the so contacted cells by morphology, mRNA expression, antigen expression or other means.

The invention also relates to a method for detecting the presence or activity of an agent which is a proliferation factor comprising the steps of:

- A. contacting the pluripotent embryonic- like stem cells of the present invention with a sample suspected of containing an agent which is a proliferation factor; and
- B. determining the proliferation and lineage of the so contacted cells by morphology, mRNA expression, antigen expression or other means;

5 wherein the proliferation of the contacted cells without lineage commitment indicates the presence or activity of a proliferation factor in said sample.

In a further aspect, the invention includes methods of testing the ability of an agent.

10 10 compound or factor to modulate the proliferation of a lineage uncommitted cell which comprises

- A. culturing the pluripotent embryonic- like stem cells of the present invention in a growth medium which maintains the stem cells as lineage uncommitted cells;
- B. adding the agent, compound or factor under test; and
- C. determining the proliferation and lineage of the so contacted cells by mRNA expression, antigen expression or other means.

The invention further relates to an assay system for screening agents, compounds or factors for the ability to modulate the proliferation of a lineage uncommitted cell, comprising:

- A. culturing the pluripotent embryonic-like stem cells of the present invention in a growth medium which maintains the stem cells as lineage uncommitted cells;
- B. adding the agent, compound or factor under test; and
- C. determining the proliferation and lineage of the so contacted cells by mRNA expression, antigen expression or other means.

25 The assay system could importantly be adapted to identify drugs or other entities that are capable of modulating the pluripotent embryonic-like stem cells of the present invention, either *in vitro* or *in vivo*. Such an assay would be useful in the development

of agents, factors or drugs that would be specific in modulating the pluripotent embryonic-like stem cells to, for instance, proliferate or to commit to a particular lineage or cell type. For example, such drugs might be used to facilitate cellular or tissue transplantation therapy.

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The assay system(s) could readily be adapted to screen, identify or characterize genes encoding proliferation or lineage-commitment factors or encoding proteins or molecules otherwise involved in cellular differentiation and development. For instance, genes encoding proteins involved in or expressed during differentiation 10 along a particular lineage could be identified by known methods (for instance cDNA libraries, differential display, etc). Thus, the pluripotent embryonic- like stem cells of the present invention could be cultured under conditions giving rise to a particular lineage and the genes therein expressed then characterized. Factors and proteins necessary for maintaining the pluripotent embryonic- like stem cells of the present 15 invention in a pluripotent embryonic-like state might also be similarly identified and characterized by culturing the pluripotent embryonic- like stem cells of the present invention under conditions maintaining their self-renewal capacity and characterizing the genes and proteins so expressed or which, when provided exogenously, will maintain the self-renewal capacity.

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In a further embodiment, the present invention relates to certain therapeutic methods which would be based upon the activity of the pluripotent embryonic-like stem cells of the present invention, including cells or tissues derived therefrom, or upon agents or other drugs determined to act on any such cells or tissues, including proliferation 25 factors and lineage-commitment factors. One exemplary therapeutic method is associated with the prevention or modulation of the manifestations of conditions causally related to or following from the lack or insufficiency of cells of a particular lineage, and comprises administering the pluripotent embryonic-like stem cells of the present invention, including cells or tissues derived therefrom, either individually or 30 in mixture with proliferation factors or lineage-commitment factors in an amount effective to prevent the development or progression of those conditions in the host.

In a further and particular aspect the present invention includes therapeutic methods, including transplantation of the pluripotent embryonic-like stem cells of the present invention, including lineage-uncommitted populations of cells, lineage-committed populations of cells, tissues and organs derived therefrom, in treatment or alleviation 5 of conditions, diseases, disorders, cellular debilitations or deficiencies which would benefit from such therapy. These methods include the replacement or replenishment of cells, tissues or organs. Such replacement or replenishment may be accomplished by transplantation of the pluripotent embryonic-like stem cells of the present invention or by transplantation of lineage-uncommitted populations of cells, lineage-committed populations of cells, tissues or organs derived therefrom.

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Thus, the present invention includes a method of transplanting pluripotent embryonic-like stem cells in a host comprising the step of introducing into the host the pluripotent embryonic-like stem cells of the present invention.

15 In a further aspect this invention provides a method of providing a host with purified pluripotent embryonic-like stem cells comprising the step of introducing into the host the pluripotent embryonic-like stem cells of the present invention.

In a still further aspect, this invention includes a method of *in vivo* administration of a protein or gene of interest comprising the step of transfecting the pluripotent embryonic-like stem cells of the present invention with a vector comprising DNA or RNA which expresses a protein or gene of interest.

5 The present invention provides a method of tissue repair or transplantation in mammals, comprising administering to a mammal a therapeutically effective amount of pluripotent embryonic-like stem cells.

10 The present invention provides a method of preventing and/or treating cellular debilitations, derangements and/or dysfunctions and/or other disease states in

mammals, comprising administering to a mammal a therapeutically effective amount of pluripotent embryonic-like stem cells.

In a further aspect, the present invention provides a method of preventing and or

5 treating cellular debilitations, derangements and or dysfunctions and or other disease states in mammals, comprising administering to a mammal a therapeutically effective amount of a endodermal, ectodermal or mesodermal lineage-committed cell derived from the pluripotent embryonic-like stem cells of the present invention.

10 The therapeutic method generally referred to herein could include the method for the treatment of various pathologies or other cellular dysfunctions and derangements by the administration of pharmaceutical compositions that may comprise proliferation factors or lineage-commitment factors, alone or in combination with the pluripotent embryonic-like stem cells of the present invention, or cells or tissues derived

15 therefrom, or other similarly effective agents, drugs or compounds identified for instance by a drug screening assay prepared and used in accordance with a further aspect of the present invention.

It is a still further object of the present invention to provide pharmaceutical

20 compositions for use in therapeutic methods which comprise or are based upon the pluripotent embryonic-like stem cells of the present invention, including lineage-uncommitted populations of cells, lineage-committed populations of cells, tissues and organs derived therefrom, along with a pharmaceutically acceptable carrier. Also contemplated are pharmaceutical compositions comprising proliferation factors or

25 lineage commitment factors that act on or modulate the pluripotent embryonic-like stem cells of the present invention and/or the cells, tissues and organs derived therefrom, along with a pharmaceutically acceptable carrier. The pharmaceutical compositions of proliferation factors or lineage commitment factors may be further comprise the pluripotent embryonic-like stem cells of the present invention, or cells,

30 tissues or organs derived therefrom. The pharmaceutical compositions may comprise

the pluripotent embryonic-like stem cells of the present invention, or cells, tissues or organs derived therefrom, in a polymeric carrier or extracellular matrix.

This invention also provides pharmaceutical compositions for the treatment of cellular debilitation, derangement and/or dysfunction in mammals, comprising:

- 5 A. a therapeutically effective amount of the pluripotent embryonic-like stem cells of the present invention; and
- B. a pharmaceutically acceptable medium or carrier.

10 Pharmaceutical compositions of the present invention also include compositions comprising endodermal, ectodermal or mesodermal lineage-committed cell(s) derived from the pluripotent embryonic-like stem cells of the present invention, and a pharmaceutically acceptable medium or carrier. Any such pharmaceutical compositions may further comprise a proliferation factor or lineage-commitment

15 factor.

The present invention relates to pluripotent stem cells capable of differentiating into cells of the mesenchymal type (PPMSCs), wherein such cells are positive for or express the antigenic markers CD10, CD13, CD34, CD56, CD90 and MHC Class-I.

20 The PPMSCs of the present invention are negative for the markers CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD11b, CD11c, CD14, CD15, CD16, CD18, CD19, CD20, CD22, CD23, CD24, CD25, CD31, CD33, CD36, CD38, CD41, CD42b, CD44, CD45, CD49d, CD55, CD57, CD59, CD61, CD62E, CD65, CD66e, CD68, CD69, CD71, CD79, CD83, CD95, CD105, CD117, CD123, CD166, Glycophorin-A.

25 DRII, FLT3, FMC-7, Annexin, and LIN.

The present invention further relates to pluripotent embryonic-like stem cells which are positive for or express the antigenic markers CD10 and CD66e and are negative for or do not express the markers CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD22, CD23, CD24, CD25, CD31, CD33, CD34, CD36, CD38, CD41, CD42b, CD44, CD45,

CD49d, CD55, CD56, CD57, CD59, CD61, CD62E, CD65, CD68, CD69, CD71, CD79, CD83, CD90, CD95, CD105, CD117, CD123, CD166, Glycophorin-A, DRII, Class-I, FLT3, FMC-7, Annexin and LIN.

5 The present invention further relates to pluripotent stem cells which are positive for or express the antigenic markers CD1a, CD10, CD41, CD66e and Annexin and are negative for or do not express the markers CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD22, CD23, CD24, CD25, CD31, CD33, CD34, CD36, CD38, CD42b, CD44, CD45, CD49d, CD55, CD56, CD57, CD59, CD61, CD62E, CD65, CD68, CD69, CD71, CD79, CD83, CD90, CD95, CD105, CD117, CD123, CD166, Glycophorin-A, DRII, Class-I, FLT3, FMC-7, and LIN.

15 The present invention also includes pluripotent stem cells which are positive for or express the antigenic markers CD1a, CD10, CD22 and Annexin and are negative for or do not express the markers CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD23, CD24, CD25, CD31, CD33, CD34, CD36, CD38, CD41, CD42b, CD44, CD45, CD49d, CD55, CD56, CD57, CD59, CD61, CD62E, CD65, CD66e, CD68, CD69, CD71, CD79, CD83, CD90, CD95, CD105, CD117, CD123, CD166, Glycophorin-A, DRII, Class-I, FLT3, FMC-7, Annexin, and LIN.

25 The present invention still further relates to pluripotent stem cells which are positive for or express the antigenic markers CD10 and CD22 and are negative for or do not express the markers CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD23, CD24, CD25, CD31, CD33, CD34, CD36, CD38, CD41, CD42b, CD44, CD45, CD49d, CD55, CD56, CD57, CD59, CD61, CD62E, CD65, CD66e, CD68, CD69, CD71, CD79, CD83, CD90, CD95, CD105, CD117, CD123, CD166, Glycophorin-A, DRII, Class-I, FLT3, FMC-7, Annexin, and LIN.

The present invention naturally contemplates several means or methods for preparation or isolation of the pluripotent embryonic-like stem cells of the present invention including as illustrated herein, and the invention is accordingly intended to cover such means or methods within its scope.

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Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A and B A. Cells isolated from adult rat marrow in primary culture 6 days after isolation. Phase contrast, 100x. Note cells in straight lines. B. Same as A. 15 Phase contrast, 200x.

FIGURE 2A-C A. Cells isolated from adult rat marrow, secondary culture, 35 days in culture. Controls. Stained with an antibody to α - myosin. Phase contrast, 100x. B. Cells isolated from adult rat marrow, secondary culture, 35 days in culture treated 20 with 10^{-7} M dexamethasone. Stained with an antibody to α - myosin. Phase contrast, 200x. Arrows point to multinucleated myotubes. C. Cells isolated from adult rat marrow, secondary culture, 35 days in culture treated with 10^{-8} M dexamethasone. Stained with an antibody to α - smooth muscle actin. Bright field, 200x. sm = smooth muscle.

25

FIGURE 3A-C A. Cells isolated from adult rat marrow, secondary culture, 35 days in culture treated with 10^{-8} M dexamethasone. Stained with Alcian blue, pH 1.0. Bright field, 100x. Arrows point to cartilage nodules. B. Cells isolated from adult rat marrow, secondary culture, 35 days in culture treated with 10^{-8} M dexamethasone. 30 Stained with Alcian blue, pH 1.0. Bright field, 200x. c = cartilage. A small myotube can be seen just below the cartilage nodule. C. Cells isolated from adult rat marrow,

secondary culture, 35 days in culture treated with 10^{-9} M dexamethasone. Stained with Von Kossa's. Bright field, 200x. Arrow points to mineral in the cartilage nodule.

5 **FIGURE 4A-C** **A.** Cells isolated from adult rat marrow, secondary culture, 35 days in culture treated with 10^{-8} M dexamethasone. Stained with Sudan Black B. Bright field, 200x. a = adipocyte. **B.** Cells isolated from adult rat marrow, secondary culture, 35 days in culture treated with 10^{-10} M dexamethasone. Stained with Von Kossa's. Bright field, 200x. b = bone. **C.** Cells isolated from adult rat marrow, secondary culture, 35 days in culture treated with 10^{-9} M dexamethasone. Stained with Von Kossa's but pretreated with EGTA. Bright field, 200x. b = bone.

FIGURE 5A and B **A.** Cells isolated from adult rat marrow, secondary culture, 35 days in culture treated with 10^{-6} M dexamethasone. Cells incubated with rhodamine-labeled acylated low density lipoprotein. Phase contrast, 100x. Arrows point to cells stained in B. **B.** Same cells as A photographed under fluorescence.

FIGURE 6A-B Phase contrast photomicrographs of primary cultures of cells isolated from day 7 wound chambers. Original magnification = 200x. **A.** Cells after 4 days in culture. **B.** Cells after 8 days in culture. Arrows point to stellate-shaped cells.

FIGURE 7A-C Secondary cultures of cells after 4 weeks in culture. **A.** Phase contrast photomicrograph of a control culture from a 7 day wound chamber stained with Alcian blue, pH 1.0. Original magnification = 200x. **B.** Phase contrast photomicrograph of an unstained culture from a day 7 wound chamber treated with 10^{-7} M dexamethasone showing multinucleated cells. Arrows point to clusters of nuclei. Original magnification = 100x. **C.** Light photomicrograph of a culture from a day 14 wound chamber treated with 10^{-7} M dexamethasone and stained with an antibody to sarcomeric myosin. Arrows point to nuclei. Original magnification = 200x.

FIGURE 8A-C Secondary cultures of cells after 5 weeks in culture. Original magnification = 200x. **A.** Phase contrast photomicrograph of a culture from a day 14 wound chamber treated with 10^{-7} M dexamethasone stained with Alcian blue, pH 1.0. c = cartilage. **B.** Phase contrast photomicrograph of a culture from day 7 wound chamber treated with 10^{-7} M dexamethasone stained with Alcian blue, pH 1.0. c = cartilage; a = adipocyte. **C.** Light photomicrograph of a culture from day 7 wound chamber treated with 10^{-7} M dexamethasone and stained with Von Kossa's. b = bone.

FIGURE 9A and B Secondary cultures of cells after 5 weeks in culture. **A.** Phase contrast photomicrograph of a culture from a day 7 wound chamber treated with 10^{-9} M dexamethasone and stained with Sudan black B. a = adipocytes. Original magnification = 200x. **B.** Light photomicrograph of a culture treated from a day 14 wound chamber with 10^{-6} M dexamethasone and stained with an antibody to smooth muscle α -actin. sm = smooth muscle. Original magnification = 100x.

FIGURE 10A and B Secondary culture of cells after 5 weeks in culture from a day 7 wound chamber treated with 10^{-6} M dexamethasone and incubated with acetylated low density lipoprotein. Original magnification = 200x **A.** Phase contrast photomicrograph. Arrows point to cells stained in B. **B.** Fluorescent photomicrograph of field shown in A. Arrows point to the same cells as in A.

FIGURE 11A-C **A.** Primary culture from 77 year old female, 5 days in culture. Phase contrast 100x. s = stellate cell m = myoblast. **B.** Primary culture from 77 year old female, 14 days in culture. Phase contrast 100x stained with antibody to myosin. **C.** Secondary culture (PPMSCs) from 77-year-old female, 35 days in culture. Phase contrast 200x.

FIGURE 12A-B **A.** Secondary culture of cells derived from 37-year-old male, 35 days in culture. Bright field 200x stained with an antibody to myosin. **B.** Secondary culture of cells derived from 37-year-old male 35 days in culture and treated with 10^{-10}

M dexamethasone. Bright field 200x stained with an antibody to myosin. Arrows point to nuclei.

FIGURE 13A-D **A.** Secondary culture derived from 77-year-old female, 28 days in culture and treated with 10^{-8} M dexamethasone. Phase contrast, 200x. Spindle shaped cells in swirl patterns. **B.** Secondary culture of cells derived from 37-year-old male, 35 days in culture, and treated with 10^{-8} M dexamethasone. Bright field, 200x stained with Alcian Blue, pH 1.0. **c** = cartilage. **C.** Secondary culture of cells derived from 37-year-old male, 35 days in culture, and treated with 10^{-8} M dexamethasone. Bright field, 200x stained with Von Kossa's stain. **b** = bone. Arrows point to adipocytes in the same culture. **D.** Secondary culture of cells derived from 37-year-old male, 35 days in culture, and treated with 10^{-7} M dexamethasone. Bright field, 200x stained with Von Kossa's stain but pretreated with EGTA. **b** = bone.

15 FIGURE 14A-C **A.** Secondary culture of cells derived from 37-year-old male, 35 days in culture, and treated with 10^{-7} M dexamethasone. Bright field, 100x stained with Sudan Black **B.** Arrows point to adipocytes. **B.** Secondary culture of cells derived from 37-year-old male, 35 days in culture, and treated with 10^{-6} M dexamethasone. Bright field, 100x and stained with antibody to smooth muscle α -actin. **sm** = smooth muscle. **C.** Same as B but shown at 200x.

FIGURE 15A and B **A.** Secondary culture of cells derived from 37-year-old male, 35 days in culture, and treated with 10^{-7} M dexamethasone. Phase contrast, 200x but cells incubated with acetylated LDL. Arrows point to cells that fluoresce in B. **B.** Same field as A but under fluorescent light. Arrows point to endothelial cells.

FIGURE 16A- B **A.** Secondary culture of cells derived from 37-year-old male, 2 days in culture, and not treated with dexamethasone (Controls). Bright field, 200x. Cells have been fixed with ethanol, are in suspension, and have been stained with an antibody to CD34. Arrows point to cells in B. **B.** Same field as A but under fluorescent light. Arrows point to cells that are CD34 positive.

FIGURE 17A-C shows 3T3 cells in secondary culture after 35 days. **A.** Control cultures, phase contrast. **B.** Culture treated with 10^{-10} M dexamethasone, phase contrast. a = adipocytes, arrows point to lipid droplets. **C.** Culture treated with 10^{-7} M dexamethasone stained with Sudan black B, bright field. a = adipocytes. Original magnification = 200x.

FIGURE 18A-C shows 3T3 cells in secondary culture. **A.** Culture treated with 10^{-3} M dexamethasone for 14 days, phase contrast. Myotube, arrows point to nuclei. **B.** Culture treated with 10^{-7} M dexamethasone for 14 days stained with a monoclonal antibody to sarcomeric myosin, bright field. Arrow points to myotube. **C.** Culture treated with 10^{-7} M dexamethasone for 14 days, phase contrast. cm = cardiac myocyte.

FIGURE 19A-C shows 3T3 cells in secondary culture after 35 days. **A.** Culture treated with 10^{-7} M dexamethasone stained with Alcian blue, bright field. c = cartilage nodule. Original magnification = 100x. **B.** Culture treated with 10^{-9} M dexamethasone stained with Alcian blue, bright field. c = cartilage nodule. Original magnification = 200x. **C.** Culture treated with 10^{-7} M dexamethasone stained with Von Kossa's stain, bright field. b = bone. Original magnification = 200x.

FIGURE 20A and B shows 3T3 cells in secondary culture after 35 days stained with a monoclonal antibody to smooth muscle α -actin. **A.** Control culture, no dexamethasone. **B.** Culture treated with 10^{-6} M dexamethasone, bright field. $sm.$ = smooth muscle cells. Original magnification = 200x.

FIGURE 21A-C shows 3T3 cells in secondary culture after 35 days, incubated with acetylated-LDL and viewed with fluorescent microscopy. **A.** Control culture, no dexamethasone. Original magnification = 100x. **B.** Culture treated with 10^{-6} M dexamethasone. Original magnification = 100x. **C.** Culture treated with 10^{-7} M dexamethasone. Original magnification = 200x.

FIGURE 22A-D. CF-SkM propagated to 30 cell doublings and incubated with insulin or dexamethasone for 0 to six weeks. Morphologies as noted. **A.** Cells treated for one week with 2 μ g/ml insulin. Note presence of four nuclei (arrows) within linear structure, indicative of a multinucleated myotube. MT. Orig. mag., 10X.

5 **B.** Cells treated for two weeks with 10⁻⁸ M dexamethasone. Note presence of clusters of cells (arrows) containing intracellular refractile vesicles indicative of adipogenic cells. Orig. mag., 10X. **C.** Cells treated for four weeks with 10⁻⁸ M dexamethasone. Note presence of nodular mass of cells with pericellular matrix halos, indicative of cartilage nodule (CN) overlying multiple multinucleated linear structures indicative of myotubes (MTs). Orig. mag., 10X. **D.** Cells treated for six weeks with 2 μ g/ml insulin. Note presence of three-dimensional matrix (delineated by arrows) overlying cell cluster, indicative of bone nodule (BN). Orig. mag., 10X.

FIGURE 23. Flow cytometry of cluster differentiation markers. "X"-axis and 15 "Y"-axis as noted on figure. NHDF propagated to 30 cell doublings and analyzed with antibodies to cell surface cluster differentiation markers.

FIGURE 24. Flow cytometry of cluster differentiation markers. "X"-axis and 20 "Y"-axis as noted on figure. NHDF propagated to 30 cell doublings and analyzed with antibodies to cell surface cluster differentiation markers.

FIGURE 25. Northern analysis of cluster differentiation markers CD10, CD13, and 25 CD56 for cell lines CF-SkM, NHDF, and PAL#3. Cells were propagated to 30 cell doublings, harvested, total RNAs extracted, electrophoresed, and probed with 32P-labeled cDNAs to CD10, CD13, CD56, and b-actin (control). As shown, mRNAs for CD13, CD56, and b-actin were being actively transcribed at time of cell harvest.

FIGURE 26A-D. NHDF propagated as noted and incubated with insulin or 10⁻¹⁰ to 30 10⁻⁶ M dexamethasone for 0 to six weeks. Morphologies as noted. **A.** Cells at 30 cell doublings post harvest treated for one week with 2 mg/ml insulin. Note presence of five nuclei (arrows) with linear structure, indicative of a multinucleated myotube. MT.

Mag. 125X. **B.** Cells at 80 cell doublings after harvest treated for two weeks with 10^{-6} M dexamethasone. Note presence of cells (arrows) containing intracellular refractile vesicles indicative of adipogenic cells. Mag., 125X. **C.** Cells at 80 cell doublings after harvest treated for four weeks with 10^{-6} M dexamethasone. Note presence of 5 nodular mass of cells with pericellular matrix halos, indicative of cartilage nodule (CN). Mag., 25X. **D.** Cells at 80 cell doublings after harvest treated for six weeks with 10^{-6} M dexamethasone. Note presence of three-dimensional matrix (delineated by arrow) overlying cell cluster, indicative of bone nodule (BN). Mag., 40X.

10 **FIGURE 27.** Flow cytometry of FSC x SSC showing R1 gated cell population of NHDF used for analysis. A similar R1 gate was used to analyze CM-SkM, CF-SkM, PAL #2, PAL #3.

15 **FIGURE 28.** Flow cytometry of cluster differentiation markers. "X"-axis denotes forward scatter (0 to 1000 linear scale) and "Y"-axis denotes side scatter (0 to 1000 linear scale). NHDF propagated to 30 cell doublings after harvest and analyzed with antibodies to cell surface cluster differentiation markers CD4 vs. CD3, CD8 vs. CD3, CD4 vs. CD8, CD34 vs. CD33, CD45 vs. CD33, CD34 vs. CD45, CD11c vs. Glycophorin-A, HLA-II (DR) vs. Glycophorin-A, and CD11c vs. HLA-II (DR).

20 **FIGURE 29.** Flow cytometry of cluster differentiation markers. "X"-axis denotes forward scatter (0 to 1000 linear scale) and "Y"-axis denotes side scatter (0 to 1000 linear scale). NHDF propagated to 30 cell doublings after harvest and analyzed with antibodies to cell surface cluster differentiation markers CD117 vs. CD36, CD45 vs. 25 CD36, CD117 vs. CD45, CD34 vs. CD90, CD45 vs. CD90, CD34 vs. CD45, CD34 vs. CD38, CD45 vs. CD38, and CD34 vs. CD45.

30 **FIGURE 30.** Northern analysis of cluster differentiation markers CD34 and CD90 for cell lines CF-SkM, NHDF, and PAL#3. Cells were propagated to 30 cell doublings after tissue harvest and released with trypsin. Total RNAs were extracted, electrophoresed, and probed with ^{32}P -labeled cDNAs to CD34, CD90, and b-actin

(control). As shown, mRNAs for CD90 and b-actin were being actively transcribed at time of cell harvest.

FIGURE 31A-C **A.** Mesenchymal stem cells isolated from 37 year old male treated with 10^{-3} M Dexamethasone, 35 days in culture. Large cell with single nucleus. Reminiscent of macrophage in culture. Phase contrast, 200x. **B.** Mesenchymal stem cells isolated from 37 year old male treated with 10^{-7} M dexamethasone, 35 days in culture. Cell with small cell body and thin, extensive cell processes. Resembles neuron in culture. Phase contrast, 200x. **C.** Mesenchymal stem cells isolated from newborn rat treated with 10^{-7} M dexamethasone, 35 days in culture. Cell looks very similar to that seen in B. Also resembles neuron in culture. Phase contrast, 200x.

FIGURE 32A-Y Human cell lines CF-NHDF2 and PAL3 incubated with insulin and/or dexamethasone for 0 to six weeks. Morphologies as noted. **A**, CF-NHDF2 treated in control medium for 24 hr, note presence of stellate-shaped mononucleated cells with large nuclear to cytoplasmic ratios, phase contrast, 200X; **B**, CF-NHDF2 treated for one week with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to myogenin (F5D), note stellate-shaped cell with intracellular cytoplasmic staining, indicative of a muscle (mesodermal) lineage, brightfield, 100X; **C**, CF-NHDF2 treated for two weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to myogenin (F5D), note binuclear and mononucleated cells with intracellular cytoplasmic staining, indicative of a muscle (mesodermal) lineage, brightfield, 100X; **D**, CF-NHDF2 treated for two weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to sarcomeric myosin (MF-20), note mononucleated cells with intracellular cytoplasmic staining, indicative of a muscle (mesodermal) phenotype, brightfield, 100X; **E**, CF-NHDF2 treated for two weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to anti-skeletal muscle fast myosin (MY-32), note mononucleated cells with intracellular cytoplasmic staining, indicative of a skeletal muscle (mesodermal) phenotype, brightfield, 100X; **F**, CF-NHDF2 treated for three weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with

antibody to anti-skeletal muscle fast myosin (MY-32), note multinucleated structure demonstrating intracellular cytoplasmic staining, indicative of a skeletal muscle (mesodermal) phenotype, brightfield, 200X; **G**, CF-NHDF2 treated for two weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to myosin heavy chain (ALD-58), note stellate structures demonstrating intracellular cytoplasmic staining, indicative of a skeletal muscle (mesodermal) phenotype, brightfield, 100X; **H**, CF-NHDF2 treated for two weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to myosin fast chain (A4.74), note stellate structures demonstrating intracellular cytoplasmic staining.

5 10 indicative of a skeletal muscle (mesodermal) phenotype, brightfield, 100X; **I**, CF-NHDF2 treated for three weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin, note linear multinucleated structure, indicative of a skeletal muscle (mesodermal) phenotype, phase contrast, 100X; **J**, CF-NHDF2 treated for six weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin, note large linear and branched

15 20 multinucleated structures, indicative of a skeletal muscle (mesodermal) phenotype, phase contrast, 100X; **K**, CF-NHDF2 treated for two weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to smooth muscle alpha-actin (1A4), note binuclear-stellate cell with intracellular cytoplasmic staining, alpha-actin intracellular staining of a binuclear-stellate is suggestive of a cardiac muscle phenotype, brightfield, 100X; **L**, CF-NHDF2 treated for two weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to smooth muscle alpha-actin (1A4), note mononuclear-stellate cells with intracellular cytoplasmic staining, smooth muscle alpha-actin intracellular staining of a mononuclear-stellate is indicative of a smooth muscle (mesodermal) phenotype, phase

25 30 contrast, 100X; **M**, PAL3 treated for four weeks with 1%, 5%, or 10% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with Sudan Black-B for saturated neutral lipids, note mononucleated cells containing intracellular-stained vesicles, indicative of an adipogenic (mesodermal) phenotype, brightfield, 100X; **N**, CF-NHDF2 treated for three weeks with 5% or 10% HS + 10^{-6} M dexamethasone and 2 ug/ml insulin and then stained with antibody to type-II pro-collagen (CIIC1), note mononuclear-stellate cell with intracellular cytoplasmic

staining, type-II procollagen intracellular staining of a mononuclear-stellate cell is indicative of a commitment to the chondrogenic (mesodermal) lineage, brightfield, 200X; **O**, CF-NHDF2 treated for three weeks with 5% or 10% HS + 10^{-6} M dexamethasone and 2 ug/ml insulin and then stained with antibody to collagen type-II (HC-II), note mononuclear-stellate cell with intracellular cytoplasmic staining, type-II collagen intracellular staining of a mononuclear-stellate cell is indicative of a commitment to the chondrogenic (mesodermal) lineage, brightfield, 100X; **P**, CF-NHDF2 treated for three weeks with 5% or 10% HS + 10^{-6} M dexamethasone and 2 ug/ml insulin and then stained with antibody to type-II collagen (D19), note

5 mononuclear-stellate cells with intracellular cytoplasmic staining, type-II collagen intracellular staining of a mononuclear-stellate is indicative of a commitment to the chondrogenic (mesodermal) lineage, brightfield, 100X; **Q**, PAL3 treated for six weeks with 5% or 10% HS + 10^{-6} M dexamethasone and 2 ug/ml insulin and then stained histochemically for chondroitin sulfate and keratan sulfate proteoglycans (Alcian

10 15 Blue, pH 1.0), dark stained nodule indicative of chondrogenic (mesodermal) phenotype, brightfield, 100X; **R**, PAL3 treated for six weeks with 5% or 10% HS + 10^{-6} M dexamethasone and 2 ug/ml insulin and then stained histochemically for chondroitin sulfate and keratan sulfate proteoglycans (Perfix/Alceec Blue), dark stained nodule indicative of chondrogenic (mesodermal) phenotype, brightfield, 50X; **S**, CF-

15 20 NHDF2 treated for two weeks with 5% or 10% HS + 10^{-6} M dexamethasone and 2 ug/ml insulin and then stained with antibody to bone sialoprotein (WV1D1), note mononuclear-stellate cells with intracellular cytoplasmic staining, bone sialoprotein intracellular staining of a mononuclear-stellate cell is indicative of commitment to the osteogenic (mesodermal) lineage, brightfield, 100X; **T**, CF-NHDF2 treated for two

20 25 weeks with 5% or 10% HS + 10^{-6} M dexamethasone and 2 ug/ml insulin and then stained with antibody to osteopontine (MP111), note mononuclear-stellate cells with intracellular cytoplasmic staining, osteopontine intracellular staining of a mononuclear-stellate cell is indicative of commitment to the osteogenic (mesodermal) lineage, brightfield, 100X; **U**, PAL3 treated for six weeks with 5% or 10% HS + 10^{-6}

25 30 M dexamethasone and 2 ug/ml insulin and then stained histochemically for calcium phosphate (von Kossa), note black-stained nodules, von Kossa-positive staining of the

three dimensional matrix of multiple nodules is indicative of an osteogenic (mesodermal) phenotype, brightfield, 50X; **V**, CF-NHDF2 treated for two weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to human-specific fibroblast specific protein (HFSP), note mononuclear-
5 stellate cells with intracellular cytoplasmic staining, fibroblast-specific protein staining of a mononuclear-stellate is indicative of a fibrogenic (mesodermal) phenotype, brightfield, 100X; **W**, CF-NHDF2 treated for two weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to peripheral endothelial cell adhesion molecule, PECAM (P2B1), note mononuclear-
10 stellate cells with intracellular cytoplasmic staining, PECAM-staining of a mononuclear-stellate is indicative of an endothelial (mesodermal) phenotype, brightfield, 200X; **X**, CF-NHDF2 treated for two weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to human-specific endothelial cell surface marker (HEndo), note mononuclear-stellate cells with
15 intracellular cytoplasmic staining, HEndo-staining of a mononuclear-stellate is indicative of an endothelial (mesodermal) phenotype, brightfield, 40X; **Y**, CF-NHDF2 treated for two weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to vascular endothelial cell adhesion molecule, VCAM (P8B1), note mononuclear-stellate cells with intracellular cytoplasmic staining,
20 VCAM-staining of a mononuclear-stellate is indicative of an endothelial (mesodermal) phenotype, brightfield, 40X.

FIGURE 33A-R Human cell line incubated with insulin and/or dexamethasone for 0 to six weeks. Morphologies as noted. **A**, CF-NHDF2 treated for two weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to selectin-E (P2H3), note mononuclear-stellate cells with intracellular cytoplasmic staining, selectin-E staining of a mononuclear-stellate is indicative of an endothelial (mesodermal) phenotype, brightfield, 100X; **B**, CF-NHDF2 treated for two weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with
25 antibody to CD34 sialomucin (CD34), note mononuclear-stellate cells with intracellular cytoplasmic staining, CD34 sialomucin-staining of a mononuclear-
30

stellate is suggestive of either an endothelial or hematopoietic (mesodermal) lineage, brightfield, 100X; **C**, CF-NHDF2 treated for four weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to neural precursor cells (FORSE-1), note mononuclear-stellate cells with intracellular cytoplasmic staining. FORSE-1 intracellular staining of mononuclear-stellate cells is indicative of commitment to the neuronal (ectodermal) lineage, brightfield, 100X; **D**, CF-NHDF2 treated for four weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to neurofilaments (RT-97), note mononuclear-stellate cells with intracellular cytoplasmic staining, neurofilament intracellular staining of 5 mononuclear-stellate cells is indicative of commitment to the neuronal (ectodermal) lineage, brightfield, 100X; **E**, CF-NHDF2 treated for four weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to neurons (8A2), note mononuclear-stellate cells with intracellular cytoplasmic staining, neuronal intracellular staining of mononuclear-stellate cells is indicative of commitment to the 10 neuronal (ectodermal) lineage, brightfield, 100X; **F**, CF-NHDF2 treated for four weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to neuroglia (CNPase), note mononuclear-stellate cells with intracellular cytoplasmic staining, neuroglial staining of mononuclear-stellate cells is indicative of commitment to the neuronal (ectodermal) lineage, brightfield, 100X; **G**, CF-NHDF2 15 treated for four weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to neurons (S-100), note mononuclear-stellate cells with intracellular cytoplasmic staining, neuronal staining of mononuclear-stellate cells is indicative of commitment to the neuronal (ectodermal) lineage, brightfield, 100X; **H**, CF-NHDF2 treated for four weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml 20 insulin and then stained with antibody to neuronal filament-200 (N-200), note mononuclear-stellate cells with intracellular neurofilament staining, neurofilament staining of mononuclear-stellate cells is indicative of commitment to the neuronal (ectodermal) lineage, brightfield, 100X; **I**, CF-NHDF2 treated for four weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to 25 human-specific nestin, a neural precursor cell marker (HNES), note mononuclear-stellate cells with intracellular cytoplasmic staining, nestin intracellular staining of 30

mononuclear-stellate cells is indicative of commitment to the neuronal (ectodermal) lineage, phase contrast, 100X; **J**, CF-NHDF2 treated for four weeks with 1% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to nestin, a neuronal precursor cell marker (MAB-353), note mononuclear-stellate cells with

5 intracellular cytoplasmic staining, nestin intracellular staining of mononuclear-stellate cells is indicative of commitment to the neuronal (ectodermal) lineage, phase contrast, 100X; **K**, CF-NHDF2 treated for two weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to keratinocytes (VM-1), note mononuclear-stellate cells with intracellular cytoplasmic staining,

10 keratinocyte-staining of a mononuclear-stellate is indicative of an epidermal (ectodermal) phenotype, brightfield, 40X.; **L**, CF-NHDF2 treated for two weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to human-specific alpha-fetoprotein (HAFP), note mononuclear-stellate cells with intracellular cytoplasmic vesicular staining, alpha-fetoprotein intracellular

15 vesicular staining of mononuclear-stellate cells is indicative of commitment to the hepatic (endodermal) lineage, brightfield, 100X; **M**, CF-NHDF2 treated for four weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to human-specific alpha-fetoprotein (HAFP), note binuclear cell with intracellular cytoplasmic vesicular staining, alpha-fetoprotein intracellular vesicular

20 staining of binuclear cell is indicative of commitment to the hepatic (endodermal) lineage, brightfield, 100X; **N**, CF-NHDF2 treated for two weeks with 1% or 5% HS + 10^{-6} M dexamethasone + 2 ug/ml insulin and then stained with antibody to human-specific epithelial-specific antigen (HESA), note mononuclear-stellate cells with intracellular cytoplasmic vesicular staining, epithelial-specific intracellular vesicular

25 staining of mononuclear-stellate cells is indicative of commitment to the epithelial (endodermal) lineage, brightfield, 100X; **O**, CF-NHDF2 treated with control media for one week and then stained with antibody to stage-specific embryonic antigen-1, SSEA-1 (MC-480), note mononuclear-stellate cells with intracellular cytoplasmic vesicular staining, SSEA-1 staining of mononuclear stellate cells is indicative of

30 embryonic stem cells, brightfield, 100X; **P**, CF-NHDF2 treated with control media for two weeks and then stained with antibody to stage-specific embryonic antigen-3,

SSEA-3 (MC-631). note mononuclear-stellate cells with intracellular cytoplasmic vesicular staining, SSEA-3 staining of mononuclear stellate cells is indicative of embryonic stem cells, brightfield, 100X; **Q**, CF-NHDF2 treated with control media for four weeks and then stained with antibody to stage-specific embryonic antigen-4,

5 **SSEA-4 (MC-813-70)**, note mononuclear-stellate cells with intracellular cytoplasmic vesicular staining, SSEA-4 staining of mononuclear stellate cells is indicative of embryonic stem cells, brightfield, 100X; and **R**, CF-NHDF2 treated with control media for six weeks and then stained with antibody to human carcinoembryonic antigen (HCEA), note mononuclear-stellate cells with intracellular cytoplasmic vesicular staining, human carcinoembryonic antigen staining of mononuclear stellate

10 cells is indicative of embryonic stem cells, brightfield, 100X.

FIGURE 34 A-R NHDF-2 Cells incubated in CM only (**A-D**) or CM plus dexamethasone (**E-R**) for 24 hr (**A**) or 56 days (**B-R**). Cells photographed at same 15 original magnification (100X) in either phase contrast (**A,L**) or bright field (**B-K,M-R**) microscopy. **A** Eight very small cells with high nuclear to cytoplasmic ratios. **B** Two very small cells heavily stained with antibody to stage-specific embryonic antigen-1 (MC480). **C** Single very small cell (arrow) stained with antibody to stage-specific embryonic antigen-3 (MC631).

20 **d** Single very small cell (arrow) heavily stained with antibody to stage-specific embryonic antigen-4 (MC813-70). **e** Four cells (arrows) stained with antibody to nestin (MAB353). **f** Four cells stained with antibody to neurons (S-100). **g** Multiple cells stained with antibody to neurofilaments (RT-97).

25 **h** Single cell with long cell processes (arrows) stained with antibody to neurofilaments (N-200). **i** Single cell stained with antibody for neuroglia (CNPase). **j** Two cells (arrows) stained with antibody for keratinocytes (VM-1). **k** Two cells (arrows) stained with antibody to myogenin (F5D).

30 **l** Two structures (arrows) containing multiple linearly arranged nuclei. **m** Multiple cells containing Oil Red-O staining intracellular vesicles.

- n Single cell stained with antibody to typ-II collagen (CIIC1).
- o Four cells (arrows) stained intracellularly with antibody to bone sialoprotein-II (WV1D1).
- p Multiple cells staining with an antibody to peripheral cel adhesion molecule 5 (PECAM, P2B1).
- q Three cells (arrows) with intracellular vesicles stained for antibody to human-specific alpha-fetoprotein (HAFP).
- r Single cell (arrow) heavily stained with antibody to human-specific gastrointestinal epithelial-specific antigen (HESA).

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FIGURE 35: Co-culture of ROSA26 PPSCs and rat astrocytes for 21 days stained with X-gal and GFAP. 100x. Cells stained with both the dark blue of the antibody 15 color reagent and blue-green of X-gal. Black arrows point to double-stained cells and white arrows to ROSA PPSCs not stained for GFAP.

FIGURE 36: Co-culture of ROSA26 PPSCs and rat astrocytes for 21 days stained with X-gal and GFAP. 40x. Can see astrocytes stained (white arrows) and then cells 20 double-stained (black arrows).

FIGURE 37: Co-culture of ROSA26 PPSCs and rat astrocytes for 21 days stained with X-gal and GFAP. 40x. White arrows point to ROSA26 PPSCs single stained for X-gal (undifferentiated) while black arrows point to ROSA cells double stained for 25 X-gal and GFAP (differentiated).

FIGURE 38: PPSCs isolated from rat skeletal muscle (RmSC-1) treated with 10-7 M dexamethasone for 21 days and then stained with anti-CNPase. 100x. White arrow points to artifact. Black arrows point to cells positive for CNPase.

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FIGURE 39: PPSCs isolated from rat skeletal muscle (RmSC-1) treated with 10-7 M dexamethasone for 21 days and then stained with antibody to IA4. Phase contrast; 100x. Black arrows point to stained cells.

5 **FIGURE 40:** PPSCs isolated from rat skeletal muscle (RmSC-1) then treated with conditioned medium from rat astrocytes for 21 days and stained with antibody RT-97. Phase contrast; 100x.

10 **FIGURE 41:** Karyotype 46, XX of CT3F cells at 37 cell doublings, isolated from a 17 year old female dermal biopsy.

FIGURE 42 depicts *in vitro* differentiation of PPSCs on Matrigel in the presence of 1% HS 10. Tube formation is evident.

15 **FIGURE 43** depicts *in vitro* differentiation of PPSCs on Matrigel in the presence of 1% HS 10 and VEGF. Tube formation is evident.

FIGURE 44 depicts PPSC localization in the bone marrow one week after IV injection into an ischemic animal.

20 **FIGURE 45** depicts ELSCs grown under different conditions 12 hours after reseeding in Matrigel.

25 **FIGURE 46** depicts ELSCs grown under different conditions after reseeding in Matrigel.

FIGURE 47 depicts ELSCs grown under different conditions after reseeding in Matrigel.

30 **FIGURE 48** depicts ELSC transplantation into a hindlimb ischemia model at 2 weeks post cell transplantation.

FIGURE 49 depicts ELSC transplantation into a hindlimb ischemia model at 2 weeks post cell transplantation.

5 **FIGURE 50** depicts muscle organogenesis by ELSCs in a hindlimb ischemia model at 2 weeks post cell transplantation.

FIGURE 51 depicts muscle organogenesis by ELSCs in a hindlimb ischemia model at 2 weeks post cell transplantation.

10 **FIGURE 52** depicts organogenesis with neovascularization by ELSC in hindlimb ischemia.

15 **FIGURE 53** depicts RT PCR results on CT3F cells. Lanes denoted 1 are of embryo body like cells reseeded for 1 week; Lanes denoted 2 are ELSCs grown in 10% HS10 serum; H are HUVEC cells.

20 **FIGURE 54** depicts RT PCR results on CT3F cells. Lanes denoted 1 are of embryo body like cells reseeded for 1 day; Lanes denoted 4 are of embryo body like cells reseeded for 4 days; Lanes denoted M are ELSCs grown in 10% HS10 serum; Lanes denoted E are ELSCs grown in 1% MCSF1 serum; B indicates brain; L indicates liver; H indicates heart.

25 **FIGURE 55** depicts RT PCR results on CT3F cells. Lanes denoted 1 are of mononuclear cells from peripheral blood; Lanes denoted 2 are of EPC cells; Lanes denoted M are ELSCs grown in 10% HS10 serum; Lanes denoted E1 and E2 are ELSCs grown in 1% MCSF1 serum; B indicates brain; L indicates liver; H indicates HUVEC cells; S indicates smooth muscle cell.

30 **FIGURE 56** depicts immunofluorescence of rat myocardium after MI and ELSC cell transplantation in rat 14.

FIGURE 57 depicts immunofluorescence of rat myocardium after MI and ELSC cell transplantation in rat 14.

5 **FIGURE 58** depicts immunofluorescence of rat myocardium after MI and ELSC cell transplantation in rat 14.

FIGURE 59 depicts immunofluorescence of rat myocardium after MI and ELSC cell transplantation in rat 14.

10 **FIGURE 60** depicts immunofluorescence of rat myocardium after MI and ELSC cell transplantation in rat 36.

FIGURE 61 depicts immunofluorescence of rat myocardium after MI and ELSC cell transplantation in rat 36.

15 **FIGURE 62** depicts immunofluorescence of rat myocardium after MI and ELSC cell transplantation in rat 14.

20 **FIGURE 63** depicts immunofluorescence of rat myocardium after MI and ELSC cell transplantation in rat 14.

FIGURE 64 depicts immunofluorescence of rat myocardium in control rat 14.

25 **FIGURE 65.** Pre and post-differentiation RMSCs morphology. **A:** RMSCs before exposure to differentiation medium: polygonal flat cells (*) predominated, with a few cells with round or triangular cell body (arrows). **B, C and D:** RMSCs morphology after 5 hours in differentiation medium; most cells had round small cell bodies and processes (B: arrows). Three of the typical morphologies of differentiated cells are shown in C and D. **C:** 90-95% of cells have small cell bodies and few processes whereas approximately 5-10% have a large round cell body and multipolar processes

30 whereas approximately 5-10% have a large round cell body and multipolar processes

(arrow). **D:** Rare cell displayed large round bodies with one or two processes and direct contact to neighboring cells (arrow). Scale: 20 μ m (A and B); 40 μ m (C and D).

5 **FIGURE 66.** Immunocytochemistry for RMSCs expanded in horse serum (**A, C, E** and **G**) or in fetal bovine serum (**B, D and F**) and differentiated for 5 hours. Immunolabeling for **A:** nestin (arrows); **B:** NF 145: Neurofilament MW 145kD (arrow). Very few cells exhibited a triangular morphology (top thin arrow); **C:** NF 200: Neurofilament MW 200kD (arrows); **D:** NSE: Neuron specific enolase (arrows); **E:** Tau positive cells with stained processes (arrows); **F:** positive (arrows) and negative (arrowheads) cells for NG2 chondroitin sulfate proteoglycan; **G:** GFAP: Glial fibrillary acidic protein (arrows). In B, C, D, E and G, asterisks are located near flat polygonal cells that are immunonegative (5-10% of all cells). **H:** Representative example of cells incubated with the secondary antibody without primary antibody.

10 **15** Scale (bar show in H for all panels): 20 μ m.

FIGURE 67. Antigenic characterization of RMSCs differentiated for 24 hours.

RMSCs expanded in HS and differentiated for 24 hours immunolabeled for **A:** NF 145: Neurofilament MW 145kD, arrows points to stained cells with processes; **C:** **20** Tau, arrows point to different positive cells with processes; **E:** NG2 immunolabeling was moderate in most cells (arrows). RMSCs expanded in FBS and differentiated for 24 hours immunolabeled for **B:** NF 145: Neurofilament MW 145kD, groups of intensely stained cells (arrows); **D:** GFAP: Glial fibrillary acidic protein (arrows). In B, C and D, asterisks are located near polygonal cells that are immunonegative. **F:** **25** Representative example of cells incubated with the secondary antibody without primary antibody. Scale (bar show in F for all panels): 20 μ m. **G:** Fluorescence assisted cell sorting (FACS) of RMSCs differentiated for 24 hours. Representative example of FACS of differentiated RMSCs immunostained with polyclonal antibodies against neuron specific enolase (NSE) (yellow), glial fibrillary acidic protein (GFAP) (**30** green), neurofilament MW 145 kDa (NF145) (blue) and NG2 chondroitin sulfate proteoglycan (NG2) (red). Cells incubated with the secondary antibody without

primary antibody were included in each experiment (black peak). Y-axis: Number of cells analyzed (events): x-axis: intensity of fluorescence staining (brightness).

FIGURE 68 shows photomicrographs from rats that were sacrificed one month (**A, C, D and E**) or two months (**B**) after transplantation. **A and B**: PPMSCs incubated with bromodeoxyuridine (BrDU) prior to transplantation and detected in tissue sections with an antibody against BrDU. Positively labeled cells were confined to the area of the injection (open arrows), and few cells have moved in the vicinity of the needle track (filled arrows). No labeled cells were found far from the needle track or in other brain regions. **C and D**: PPMSC transfected with a vector expressing Green Fluorescence Protein (GFP). **C**: Low magnification photomicrograph showing the transplant with numerous fluorescent cells. Some cells with processes expressing GFP were observed at high magnification (**D**: arrows). **E**: PPMSCs transfected with beta-galactosidase (β -Gal): the cells were visualized one month post-transplantation with histochemistry. The cells did not migrate from the injection area, but were localized in the needle track (open arrow) or its vicinity (filled arrow).

FIGURE 69 depicts immunofluorescence of rat A2B2 scl-40 cells stained with various antibodies.

FIGURE 70 depicts immunofluorescence of rat A2B2 scl-40 cells stained with various antibodies.

FIGURE 71 depicts immunofluorescence of ROSA ELSC cells stained with various antibodies.

FIGURE 72 depicts immunofluorescence of ROSA ELSC cells stained with various antibodies.

FIGURE 73. Femoropatellar groove, 26 weeks post- op. Empty defects, 40x, stained with Toluidine blue. **A.** Animal #70. **B.** Animal # 74.

FIGURE 74. Femoropatellar groove, 26 weeks post-op. Defects with polymer alone, 40x. **A.** Stained with Mallory-Heidenhain, animal # 71. **B.** Stained with Toluidine blue, animal # 72.

5 **FIGURE 75.** Femoropatellar groove, 26 weeks post-op. Defects with PPSCs cultured in the polymer for 24 hours prior to implantation, 40x. Stained with Toluidine blue. **A.** Animal #71. **B.** Animal #79. Fat can be seen at the left hand side of panel A where the center of the defect would be.

10 **FIGURE 76.** Femoropatellar groove, 26 weeks post-op. Defects with PPSCs cultured in the polymer for 2 weeks prior to implantation, 40x. Stained with Toluidine blue, animal #69.

15 **FIGURE 77.** Medial condyle, 26 weeks post-op. Empty defect, 40x, stained with Toluidine blue. **A.** Animal #70. **B.** Animal #75.

FIGURE 78. Medial condyle, 26 weeks post-op. Defect with polymer alone, 40x, stained with Toluidine blue, animal #71.

20 **FIGURE 79.** Medial condyle, 26 weeks post-op. Defects with PPSCs cultured in the polymer for 24 hours prior to implantation, 40x, stained with Toluidine blue. **A.** Animal #71. **B.** Animal #79.

25 **FIGURE 80.** Medial condyle, 26 weeks post-op. Defects with PPSCs cultured in the polymer for 2 weeks prior to implantation, 40x, stained with Toluidine blue. **A.** Animal #64. **B.** Animal #74.

DETAILED DESCRIPTION